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The Enteropathogenic *Escherichia coli* Effector Cif Induces Delayed Apoptosis in Epithelial Cells[▽]

Ascel Samba-Louaka,[1,2] Jean-Philippe Nougayrède,[1,2] Claude Watrin,[1,2] Eric Oswald,[1,2,3,4] and Frédéric Taieb[1,2]*

INRA, UMR1225, F-31076 Toulouse, France[1]; Université de Toulouse, ENVT, UMR 1225, F-31076 Toulouse, France[2]; Université de Toulouse, UPS, Faculté de Médecine, Toulouse F-31400[3]; and Laboratoire de Bactériologie-Hygiene, CHU de Toulouse, Institut Fédératif de Biologie, Toulouse F-31059, France[4]

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The cycle inhibiting factor (Cif) belongs to a family of bacterial toxins, the cyclomodulins, which modulate the host cell cycle. Upon injection into the host cell by the type III secretion system of enteropathogenic *Escherichia coli* (EPEC), Cif induces both G_2 and G_1 cell cycle arrests. The cell cycle arrests correlate with the accumulation of $p21^{waf1}$ and $p27^{kip1}$ proteins that inhibit CDK-cyclin complexes, whose activation is required for G_1/S and G_2/M transitions. Increases of p21 and p27 levels are independent of p53 transcriptional induction and result from protein stabilization through inhibition of the ubiquitin/proteasome degradation pathway. In this study, we show that Cif not only induces cell cycle arrest but also eventually provokes a delayed cell death. Indeed, 48 h after infection with EPEC expressing Cif, cultured IEC-6 intestinal cells were positive for extracellular binding of annexin V and exhibited high levels of cleaved caspase-3 and lactate dehydrogenase release, indicating evidence of apoptosis. Cif was necessary and sufficient for inducing this late apoptosis, and the cysteine residue of the catalytic site was required for Cif activity. These results highlight a more complex role of Cif than previously thought, as a cyclomodulin but also as an apoptosis inducer.

Enteropathogenic *Escherichia coli* (EPEC) constitutes a major cause of severe infant diarrhea in developing countries (25). Infection of intestinal epithelial cells with EPEC produces a characteristic histopathological feature known as an "attaching and effacing" (A/E) lesion. This lesion is characterized by intimate bacterial attachment, formation of an actin-rich pedestal structure, and localized destruction of the brush border microvilli (15, 16). This bacterial attachment is detected in vitro through the use of the fluorescent actin staining test (15). The genes required for the formation of A/E lesions are clustered on the pathogenicity island named the locus of enterocyte effacement (LEE), which codes for a type III secretion system (T3SS), a molecular syringe that allows translocation into the host cell of up to 40 effector proteins that subvert eukaryotic cellular pathways for the pathogen's benefit (20, 21). The LEE does not carry all genes necessary for EPEC virulence. Indeed, the cycle inhibiting factor (Cif) belongs to a repertoire of proteins that use the T3SS to be injected into the host cell (19). The *cif* gene is located on a lambdoid prophage found in the chromosomes of some EPEC and enterohemorrhagic *E. coli* strains (18, 19). The Cif protein is composed of an exchangeable N-terminal domain that is necessary for its secretion and translocation through the T3SS (2). Cif displays substantial structural and functional homology with four putative proteins found in pathogenic and symbiotic bacteria. The crystal structures of different homologs of Cif and the presence of a conserved catalytic triad (Cys109-His165-Gln185) suggest that Cif belongs to a superfamily of cysteine proteases, transglutaminases, and acetyltransferases (5, 12, 13, 35). Upon translocation in HeLa cells, Cif triggers a cytopathic effect characterized by stress fiber and focal adhesion formation and by cell cycle arrest at both G_1/S and G_2/M transitions, depending on the stage of cells in the cell cycle during infection (19, 26, 32). The cytostatic effect induced by Cif is independent of the cell type and p53 status (34) and is correlated with stabilization of $p21^{waf1}$ and $p27^{kip1}$ proteins that regulate the host cell cycle (32). Any mutation of the residues of the catalytic triad abrogates the Cif-associated cytopathic effect and suppresses p21 and p27 accumulations, confirming that the activity of Cif is dependent on the intact enzymatic site of the protein (13, 15, 34).

In the present study, we investigated the fate of untransformed intestinal epithelial (IEC-6) cells exposed to Cif protein beyond cell cycle arrest. We demonstrated that Cif induced a cell death corresponding to apoptosis. This effect was a late event and needed a functional catalytic site of Cif. The cytopathic effect of Cif in vitro could consist of two steps, namely, cell cycle arrest and, eventually, cell death.

MATERIALS AND METHODS

Cell lines, bacterial strains, and plasmids. Small intestine epithelial cells from *Rattus norvegicus* IEC-6 (CRL-1592) were maintained in Dulbecco's modified Eagle's medium (DMEM GlutaMax; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 80 μg ml^{-1} gentamicin, and bovine insulin (0.1 unit ml^{-1}; Sigma) at 37°C in a 5% CO_2 atmosphere.

The EPEC strains used were rabbit EPEC O103 strain E22 and human EPEC O111 strain B171-8. The mutant strains E22 *cif*::*frt* (E22ΔCif) and B171 *cif*::*frt* (B171ΔCif) were previously described (19, 32, 34).

Plasmids pEL3 ($pCif_{wt}$) and pGJ715 ($pCif_{C109A}$) were previously described (32).

Infection and cell treatments. For infection experiments, bacterial strains were cultured overnight in Luria-Bertani (LB) broth and then diluted 1:20 in DMEM supplemented with 25 mM HEPES and 5% FBS for 2 h at 37°C. IEC-6 cells were

* Corresponding author. Mailing address: Université de Toulouse, ENVT, UMR 1225, F-31076 Toulouse, France. Phone and fax: 33 5 61 19 32 86. E-mail: f.taieb@envt.fr.

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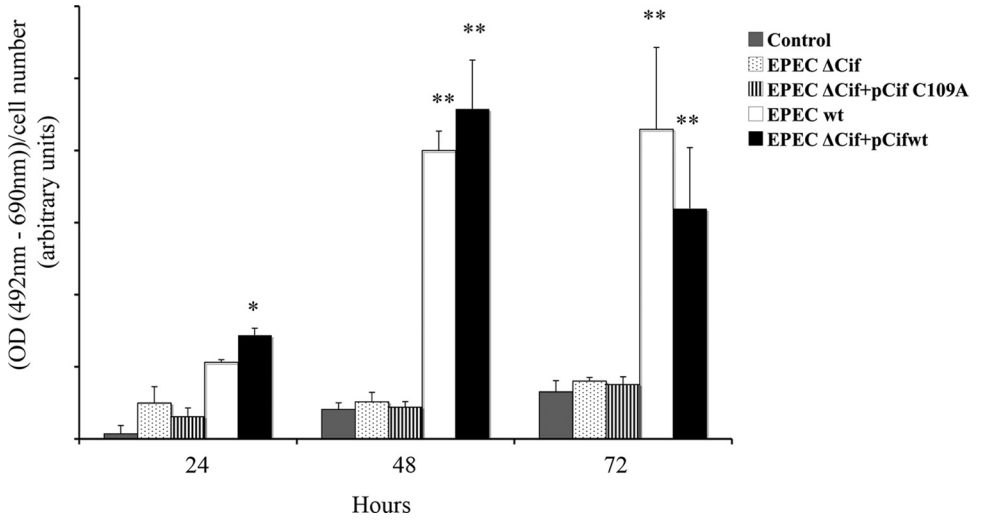

FIG. 1. EPEC E22 strains expressing Cif induce the death of IEC-6 cells. Cells were infected for 2 h with EPECwt, EPECΔCif, or EPECΔCif complemented with plasmids coding for wild-type Cif (pCifwt) or a Cif C109A mutant (pCifC109A). Twenty-four, 48, and 72 hours after infection, the culture supernatants were collected to measure the LDH activity. Graphed data represent the means plus standard errors of the means for three independent experiments. Data are significantly different (*, $P < 0.05$; **, $P < 0.001$) compared with the control for each time point.

washed three times in Hanks' balanced salt solution (Invitrogen) and infected for 2 h in DMEM supplemented with 25 mM HEPES and 5% FBS with a multiplicity of infection of 50 bacteria per cell. After infection, cells were washed three times in Hanks' balanced salt solution and incubated in the growth medium supplemented with 200 μg ml^{-1} gentamicin.

For BioPORTER assays (Genlantis), 80 μl (250 μg ml^{-1}) of purified Cif (or phosphate-buffered saline [PBS] as a negative control) was added to one BioPORTER tube and resuspended in 920 μl of DMEM. The samples were added to the cells for 4 h before being replaced by fresh growth medium.

Immunofluorescence microscopy. Cells cultured in chamber slides (Labtek; Becton Dickinson) were fixed with 3.6% formaldehyde for 15 min at room temperature and were permeabilized for 5 min in PBS with 0.1% Triton X-100. Cells then were incubated with rhodamine-phalloidin (Molecular Probes) to stain F-actin and with DAPI (4′,6-diamidino-2-phenylindole; Sigma) to stain DNA. Images were acquired with a DMRB fluorescence microscope equipped with a DFC300FX digital camera (Leica).

LDH release assay. Twenty-four, 48, and 72 hours after infection, the supernatant medium from infected cells was collected and the amount of lactate dehydrogenase (LDH) released was measured with a cytotoxicity detection kit from Roche. Absorbances at 492 nm and 690 nm were measured in a microplate reader (Tecan Infinite M200). The number of cells was evaluated by manual counting under a microscope. We determined the ratio between the raw absorbance data and cell number to integrate the effect of cell number on the level of LDH released.

Assay for quantification of exposure of phosphatidylserine on the outer leaflet. Cells were treated with annexin V conjugated to fluorescein isothiocyanate (FITC) according to the manufacturer's recommendations (annexin V-FITC; Miltenyi Biotec). Briefly, 10^6 cells were washed with binding buffer by centrifugation at 300 × *g* for 10 min. Ten microliters of annexin V-FITC was added to 100 μl of cell suspension for 15 min at room temperature. Cells were washed again and incubated with 0.5 μg of propidium iodide (PI). Acquisitions were performed on a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed using FlowJo software (Tree Star). Cells with intact membranes do not incorporate PI. Cells in late apoptosis or necrosis lost membrane integrity and retained both annexin V and PI; viable cells were negative for both stains, and early apoptotic cells were positive for annexin V and negative for PI staining.

Assessment of caspase activation in IEC-6 cells. We evaluated caspase activation in cells by use of a kit (CaspaTag pan-caspase in situ assay kit, fluorescein; Chemicon International) which uses a peptide inhibitor of caspase that produces green fluorescence. IEC-6 cells were infected or not (control) for 2 h with a wild-type EPEC E22 strain (EPECwt), a strain deleted for the *cif* gene (EPECΔCif), or a strain complemented with a plasmid coding for Cif (EPECΔCif +pCifwt). According to the manufacturer's recommendations, cells were treated with the FLICA (fluorochrome inhibitors of caspases) reagent for 1 h at 37°C under 5% CO_2. After four washes, cells were resuspended in washing buffer, measurements were performed on a FACSCalibur flow cytometer (Becton Dickinson), and data were analyzed using FlowJo software (Tree Star).

Western blot analysis. For Western blot analyses, approximately 6×10^5 cells were lysed in 80 μl of Laemmli loading buffer (Bio-Rad), sonicated for 2 s to shear DNA, and then boiled for 5 min. Proteins were resolved in 4 to 12% NuPage gradient gels (Invitrogen) and transferred to polyvinylidene difluoride membranes. The membranes were saturated in TBST (10 mM Tris, pH 7.8, 150 mM NaCl, 0.1% Tween 20) with 10% nonfat dry milk and then probed with primary antibodies in TBST with 5% nonfat dry milk. Primary antibodies were anti-actin (ICN), anti-cleaved caspase-3 (Cell Signaling Technology), and anti-p21 (Santa Cruz Biotechnology). Bound antibodies were visualized with horseradish peroxidase-conjugated secondary antibody. Acquisitions were performed with a ChemiDoc XRS molecular imager (Bio-Rad).

Statistical analysis. Analyses were performed by two-way analysis of variance with the Bonferroni posttest (GraphPad Prism IV software). Statistical significance was established at *P* values of <0.05.

RESULTS

EPEC adheres to IEC-6 cells and forms A/E lesions. Prior to studying the effects of EPEC infection on intestinal IEC-6 cells, we assessed the potential of EPEC to induce a fluorescence actin staining effect that testifies to the presence of a functional T3SS and effective bacterial adhesion. Cells infected with wild-type EPEC displayed spots of fluorescence of actin that corresponded to attachment of bacteria to the cells (15). This phenotype did not depend on the *cif* gene, since its mutation did not impair the ability of bacteria to adhere to cells (data not shown). As expected, strains mutated in the *escN* gene (type III secretion deficient) were unable to induce such a pattern of actin condensation (data not shown).

EPEC strains expressing Cif induce delayed cell death of host cells. Our previous work demonstrated that infection of cells with bacteria expressing Cif leads to cell cycle arrests (19, 32). In order to evaluate the outcome of arrested cells, we examined their viability through the release of LDH. LDH is released from dying cells in the cell medium. As shown in Fig. 1, noninfected (control) cells released a basal level of LDH after 24 h that could have resulted from cell death at confluence. In contrast, we observed a high level of release of LDH

TABLE 1. Percentage of cells positive for activation of caspases[a]

Strain	% of cells positive for caspase cleavage		
	24 h	48 h	72 h
Control	5.0	5.9	5.4
EPECwt	11.3	18.3	14.7
EPECΔCif	5.0	8.6	6.5
EPECΔCif+pCifwt	10.5	18.9	13.6

[a] See Materials and Methods for details.

from cells infected with EPECwt. This effect was more pronounced 48 h and 72 h after infection. The Cif protein was specifically implicated in the induction of cell death, since its deletion (EPECΔCif) suppressed LDH release. Cell death was restored by complementation with a *cif*-carrying plasmid (EPECΔCif+pCif$_{wt}$). However, complementation with a plasmid encoding Cif mutated at its catalytic cysteine (EPECΔCif+pCif$_{C109A}$) did not provoke the LDH release observed with wild-type Cif (Fig. 1). This result shows that Cif requires an intact catalytic site to induce delayed cell death in IEC-6 cells.

EPEC strains expressing Cif induce apoptosis of IEC-6 cells. The release of LDH by IEC-6 cells following infection could result from oncosis (necrosis) or secondary necrosis (a process that occurs in vitro, in which late-stage apoptotic cells that fail to be engulfed by phagocytes undergo necrosis) (33). In order to assess the type of cell death induced by Cif, we used FLICA to quantify apoptosis through detection of activated caspases. As in control cells, infection by EPECΔCif led to a percentage of cells with activated caspases of about 5%. However, EPECwt infection induced a substantial increase of caspase-activated cells that reached a maximum at 48 h. The complemented strain induced a similar increase, strengthening the role of Cif in caspase activation (Table 1). As with LDH, the level of activation of caspases was more pronounced 48 h after infection.

We further analyzed the accumulation of cleaved caspase-3 that is characteristic of apoptotic cells (17). Infection with EPEC expressing wild-type Cif led to an accumulation of

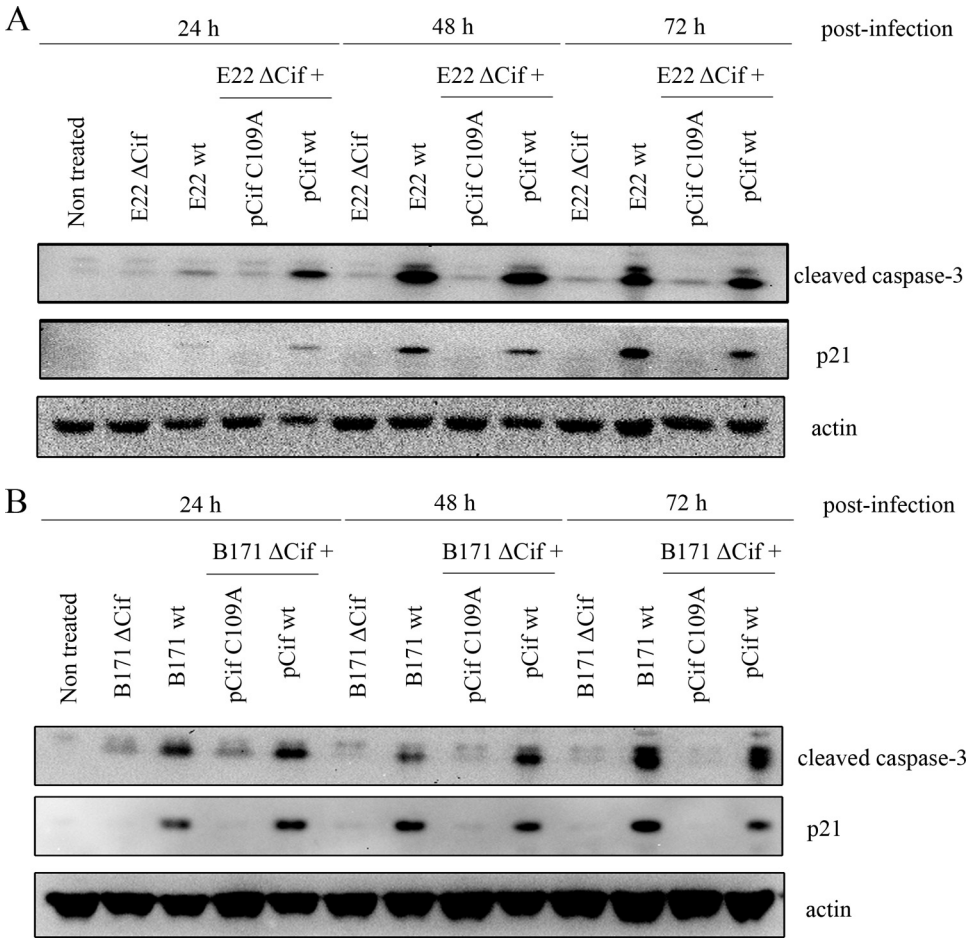

FIG. 2. EPEC strains induce accumulation of cleaved caspase-3 in IEC-6 cells. (A) IEC-6 cells were infected for 2 h with an EPEC E22 wild-type strain (E22wt), a Cif-deleted mutant (E22ΔCif), or E22ΔCif complemented with a plasmid coding for wild-type Cif (pCif wt) or a Cif C109A mutant. After the indicated times, cell extracts were probed with anti-p21, anti-cleaved caspase-3, and anti-actin antibodies. (B) The same experiment was done with an EPEC B171-8 wild-type strain (B171wt), a Cif-deleted mutant (B171ΔCif), or B171ΔCif complemented with a plasmid coding for wild-type Cif (pCif wt) or a Cif C109A mutant.

cleaved caspase-3. This accumulation correlated with the Cif-dependent increase of the level of $p21^{waf1}$ (Fig. 2A) (32). Twenty-four hours after infection, the level of cleaved caspase-3 in cells infected with EPECwt was lower than that in EPECΔCif+pCif_{wt}-infected cells, probably reflecting a dose effect of Cif, since pCif_{wt} is a multicopy plasmid. In both cases, a maximum level of cleaved caspase-3 was reached 48 h after infection. The apoptosis observed was not restricted to the rabbit EPEC E22 strain. We also observed an accumulation of cleaved caspase-3 with the typical human EPEC strain B171-8 (Fig. 2B). In contrast to infection with the E22 strain, a high level of accumulation of cleaved caspase-3 was obtained as soon as 24 h after infection with B171wt.

Another feature of apoptosis is phosphatidylserine exposure on the outer leaflet of the plasma membrane. As shown in Fig. 3A, early apoptotic cells are positive for annexin V (which binds phosphatidylserine) and negative for PI staining (indicating that the plasma membrane is not breached). The percentage of apoptotic cells obtained with E22ΔCif was similar to that for control cells and increased when infection was done with the wild-type strain. This effect was more pronounced with E22ΔCif+pCif_{wt} and reached a maximum at 48 h postinfection (Fig. 3B). Infection with EPEC B171 strains also led to a significantly higher percentage of apoptotic cells when the bacteria expressed wild-type Cif than with B171ΔCif and B171ΔCif+pCif_{C109A} 24 h, 48 h, and 72 h following infection (Fig. 3C). However, several differences were observed between the two EPEC strains. B171ΔCif but not E22ΔCif induced a significantly higher phosphatidylserine exposure level than that of control cells, indicating a Cif-independent proapoptotic activity in strain B171. Overexpression of Cif by B171ΔCif+pCif_{wt} induced the highest percentage of cells with phosphatidylserine exposed at 24 h, which decreased 48 h and 72 h after infection (Fig. 3C). Thus, the kinetics of apoptosis were different between cells infected by E22 or B171. Such differences could be explained partially by the different sets of effectors expressed by these two EPEC strains.

Cif is sufficient to induce apoptosis of IEC-6 cells. It was reported that different EPEC proteins could modulate cell death, such as the bundle-forming pilus (BFP) in strain B171 (1) or the EspF effector (4). To ensure that Cif alone was able to induce apoptosis, we delivered His-tag-purified Cif or Cif C109A into cells through a lipid-based protein delivery system (BioPORTER) (34). The activity of Cif was confirmed by accumulation of $p21^{waf1}$ protein (Fig. 4). Cif induced an accumulation of cleaved caspase-3 48 h after infection. This effect was dependent on the Cif catalytic domain, since the level of cleaved caspase-3 induced by Cif C109A was lower than that with wild-type Cif (Fig. 4). The moderate increases of the cleaved caspase-3 level observed in nontreated cells and in those treated with BioPORTER+PBS or BioPORTER+Cif C109A were likely due to cell confluence. Together, these results demonstrate that Cif does not rely on EPEC cofactors to induce delayed apoptosis.

DISCUSSION

The EPEC effector Cif induces cell cycle arrest at both G_1/S and G_2/M of host cells (19, 32). Thus, Cif is one of the inhibitory cyclomodulins, a growing family of bacterial effectors and toxins that modulate the eukaryotic cell cycle (28, 29). This cytostatic effect is correlated with stabilization of $p21^{waf1}$ and $p27^{kip1}$ cell cycle inhibitors (32). Since the arrest of cell proliferation by Cif is irreversible, we investigated the fate of cells exposed to Cif. We found that in addition to its inhibitory cyclomodulin activity, Cif induced a cell death of host cells characterized by activation of caspases, accumulation of cleaved caspase-3, exposure of the phosphatidylserine on the outer leaflet, and LDH release. These features are consistent with apoptosis and occurred 2 days after infection. Previous studies on Cif were mostly performed using the handy HeLa cell model and showed that Cif induces cell cycle arrest and rereplication for up to 72 h after infection. However, cell death was also evident in HeLa cells after 72 h postinfection (data not shown). In this study, we used intestinal epithelial IEC-6 cells, which are more relevant to the natural niche of EPEC infection. In contrast to HeLa cells, this nontransformed cell line is p53 positive. Since p53 is implicated in the apoptosis pathway (22), the difference in kinetics of cell death between these two cell lines is very likely dependent on the genetic background of IEC-6 and HeLa cells, at least concerning p53. The effect of Cif could consist of stopping the cell cycle before killing the host cell. This kind of effect is reminiscent of the DNA damage response. This checkpoint activation leads to cell cycle arrest to allow repairs of injury, and if damages persist, cells undergo a cell death process by activation of proapoptotic genes (23, 31). However, neither DNA damage nor the activation of the DNA damage response was detected in cells in contact with Cif (34). Both cell cycle arrest and apoptosis could also result from inhibition of the proteasomal degradation pathway. Indeed, the proteasome inhibitor lactacystin induces cell cycle arrest at both G_1/S and G_2/M and induces apoptosis of treated cells (7–9, 14). Since we found that Cif modulates protein stability through inhibition of the ubiquitin/proteasome pathway as specifically as lactacystin (32), such a Cif-subverted pathway is likely to eventually lead IEC-6 cells to cell death.

The induction of apoptosis by EspF of EPEC on epithelial cells, early after infection, has been reported (3, 4, 27). This effect was independent of Cif, since the EPEC reference strain E2348/69 possesses a mutated *cif* gene (19). In contrast to Cif, the effect of EspF on apoptosis occurs just after infection and persists until 6 h postinfection. Also, Crane et al. found that all cells transfected with *espF* were killed after 48 h (4). However, infection of untransformed IEC-6 cells could induce a different response compared to HeLa or T84 cell lines. Indeed, Nagai et al. observed that in contrast to the case for HeLa cells, the LDH assay conducted on T84 cell monolayers gives no significant differences in cytotoxicity induced by EPECwt and a Δ*espF* mutant (24).

Another EPEC feature implicated in apoptosis of host cells relies on the presence of BFP. As with EspF, BFP induces an early cell death as soon as 5 h postinfection (1). In our study, we observed different responses in induction of phosphatidylserine exposure when we infected cells with atypical E22 (*bfp*-negative) and typical B171-8 (*bfp*-positive) EPEC strains (Fig. 3C). The presence of BFP could have led to the high annexin V staining level (phosphatidylserine exposure) observed 24 h after infection with the B171 strain, regardless of the presence

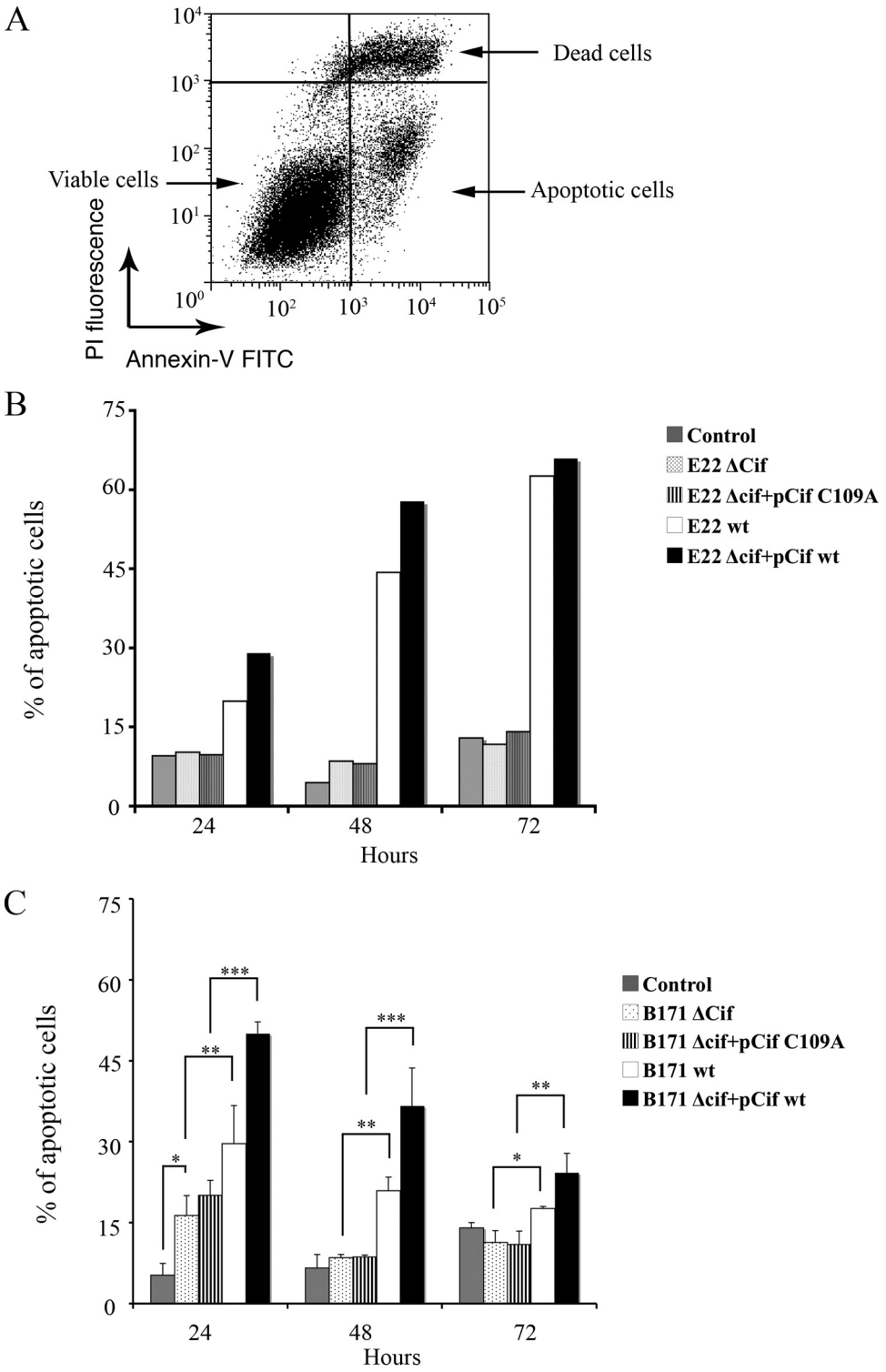

FIG. 3. Cif induces exposure of phosphatidylserine on infected IEC-6 cells. (A) IEC-6 cells were infected with the EPEC B171ΔCif strain. Twenty-four hours after infection, phosphatidylserine exposure was analyzed by flow cytometry as described in Materials and Methods. Viable, dead, and apoptotic cell populations are indicated. (B) Cells were infected or not (control) with the indicated EPEC E22 strains and incubated for 24 h, 48 h, and 72 h. Percentages of apoptotic cells (lower right quadrant in panel A) were measured. The experiment was performed twice with similar results, and data for only one experiment are shown. (C) Cells were infected with the indicated EPEC B171 strains. The experiment was performed three times, and the results are shown as means ± standard errors of the means. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

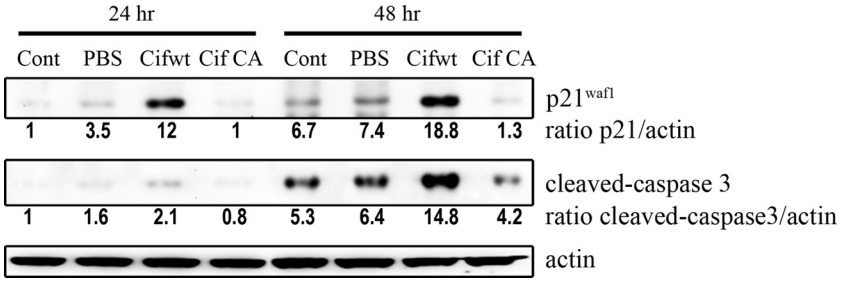

FIG. 4. Purified Cif induces apoptosis in IEC-6 cells. IEC-6 cells were left untreated (cont) or were incubated with BioPORTER plus PBS, H6-Cifwt, or H6-Cif C109A for 24 h and 48 h, and cellular extracts were probed with the indicated antibodies. p21 and cleaved caspase-3 levels were quantified by densitometry with Quantity One software (Bio-Rad) and normalized to the actin level. Relative levels of p21 and cleaved caspase-3 are shown as increases compared to those in control cells at 24 h.

of Cif (Fig. 3C). However, the effect of Cif on the induction of apoptosis was still observed within this *bfp*-positive bacterial strain. Moreover, since delivery of purified Cif into the host cell by BioPORTER was able to induce cleavage of caspase-3 (Fig. 4), we demonstrated that Cif alone was sufficient to induce apoptosis, independently of EspF, BFP, or other bacterial factors.

The role of induction of apoptosis in EPEC pathogenesis remains difficult to understand. A recent study reported that induction of caspases was dispensable to increase paracellular permeability in vivo (10). Another study revealed the ability of the EspF effector produced by *Citrobacter rodentium* to induce cell death of intestinal cells in mice, which correlates with a high mortality compared to that with an EspF mutant (24). Our future work will evaluate the induction of apoptosis by Cif in vivo. Indeed, expression of Cif or its mode of action could be influenced by the presence of other effectors (6). The induction of $p21^{waf1}$ by Cif in vivo must also be studied. During the renewal of intestinal epithelium, cells migrate to the lumen by proliferating, differentiating before being extruded after apoptosis (30). Several pieces of data report that $p21^{waf1}$ could induce or inhibit apoptosis (11). Thus, through $p21^{waf1}$, Cif could inhibit cell turnover of the intestinal epithelium by preventing proliferation and/or by inducing apoptosis and thus favoring bacterial colonization. While the role of Cif in bacterial virulence remains to be understood, this study highlights a new facet of the cyclomodulin's properties, as an apoptosis inducer.

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